SODIUM CHANNEL ALPHA SUBUNIT VARIANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application Ser. No. 60/401,018, filed on August 2, 2002, which is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with United States government support awarded by the following agency: NIH, Grant No. HL56441. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Sodium channel proteins embedded in cellular membranes of muscle cells, neurons and other excitable cells help produce and propagate electrical impulses and are implicated in many human diseases and conditions. Sodium channels are often composed of a pore-forming α subunit, having four homologous domains DI-DIV and six transmembrane regions S1-S6 per domain, and at least three auxiliary subunits β1, β2 and β3. The α subunit is sufficient to form a functional channel for generating sodium current flow across cellular membranes. An extensive review of cardiac ion channels published in Annual Review of Physiology 64:431-75 (2002) is incorporated by reference in its entirety as if set forth herein.

[0004] Human cardiac sodium channels play a critical role in cardiac excitation. $hNa_v1.5$, a human cardiac sodium channel α subunit encoded by the *SCN5A* gene forms a functioning monomeric sodium channel that carries the inward Na current (I_{Na}) in the heart. The I_{Na} current is vital for excitation and conduction in working myocardium and in specialized conduction tissue such as Purkinje fibers.

[0005] Three distinct full-length polymorphic *SCN5A* clones that encode the hNav1.5 human cardiac sodium channel (designated *SCN5A* hH1, *SCN5A* hH1a, and *SCN5A* hH1b (or simply

hH1, hH1a, and hH1b, respectively) have been isolated from human cardiac cDNA libraries (Gellens, M.E. et al., "Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel," *Proc.Natl.Acad.Sci. U.S.A.* 89, 554-558 (1992); Hartmann, H. A. et al., Effects of III-IV linker mutations on human heart Na^+ channel inactivation gating. *Circ.Res.* 75, 114-122 (1994), Ye, B. and J. Makielski, Third Complete Sequence of Human Cardiac Sodium Channel α Subunit Reveals Polymorphism in Domain I and II, Biophys. J. 80(1):225c (2001), each incorporated by reference herein as if set forth in its entirety.).

[0006] Subsequent to the publication of the hH1b sequence by Ye and Makielski, the authors determined errors in the published *SCN5A* hH1 protein sequence. The true polymorphisms among hH1, hH1a and hH1b are reflected in Table 1, *infra*. The amino acid numbering follows that of the original hH1 clone which contains 2016 amino acids. All of the differences are confined to the cytoplasmic linkers between DI-II and between DII-III. Briefly, hH1 and hH1a differ by just 3 amino acids --T559 vs. A559, Q1027 vs. R1027, and Q1077 vs. Q1077del (hH1 vs. hH1a, respectively) -- over a total length of 2016 / 2015 amino acids, respectively. The hH1b protein also differs from either hH1 or hH1a at positions 559, 1027 and 1077, as well as at positions 558 and 618. The arginine at position 558 in hH1b is consistent with a previously characterized histidine-to-arginine polymorphism (Iwasa, et al., "Twenty single nucleotide polymorphisms (SNPs) and their allelic frequencies in four genes that are responsible for familial long QT syndrome in the Japanese population," *J.Hum.Genet.* 45, 182-183). The isoleucine at position 618 is consistent with a known high-frequency spontaneous conservative leucine-to-isoleucine substitution.

[0007] The significance of polymorphisms in the sodium channel is still unknown. For example, it is not known how such polymorphisms affect the mutation phenotype of *SCN5A*. Nonetheless, identified polymorphisms can help identify disease-associated mutations in *SCN5A*. For example, various *SCN5A* mutations are associated with congenital Long QT syndrome, idiopathic ventricular fibrillation and the Brugada syndrome (Keating and Sanguinetti 2001).

[0008] In separate studies, the two known polymorphic forms showed only minor kinetic differences that can be attributed to different expression systems and study techniques including solutions, temperature, and protocols. (Gellens, M.E. et al., *supra*; Hartmann et. al., *supra*; and Wattanasirichaigoon et. al. 1999). Subtle differences in kinetics such as decay rates, inactivation midpoints, and late I_{Na}, however, may be important in controlling repolarization.

[0009] Sodium channel α subunits encoded by an *SCN5A* hH1a clone carrying an arrhythmogenic missense methionine-to-leucine mutation at amino acid 1766 (M1766L) further exhibit a significant inward sodium current level drop, relative to the current level in channels encoded by a wild type hH1a clone. Recently, M1766L in the hH1a background was shown to have a trafficking defect and to cause QT prolongation and ventricular arrhythmia. These conditions can be rescued by low temperature, antiarrhythmic drugs and the β1 subunit. Valdivia et. al., C. R. et al., A Novel SCN5A Arrhythmia Mutation M1766L with Expression Defect Rescued by Mexiletine," *Cardiovasc. Res.* 54(3):624-9 (2002).

[0010] In another aspect, drugs that can alter sodium channel activities can relieve or prevent symptoms of certain conditions such as cardiac arrhythmias. Cardiac arrhythmias are abnormalities in the rate, regularity, or site of origin of the cardiac impulse, or a disturbance in conduction of the impulse that alters the normal sequence of atrial or ventricular activation. One known way to treat cardiac arrhythmias is to block the activity of a cardiac sodium channel. Sodium channel blockers used to treat cardiac arrhythmias include: Quinidine, Lidocaine, Procainamide, Mexiletine, Flecainide, Moricizine, and Disopyramide. Identifying other polymorphic forms of human cardiac sodium channel will advance our understanding of sodium channel-related heart problems and provide new tools for developing diagnostic, prophylactic and therapeutic strategies.

[0011] The art is uncertain as to whether any of the three polymorphic *SCN5A* isolates encodes a standard or reference $hNa_v1.5$ protein. This uncertainty precludes a well-reasoned analysis of mutations at particular amino acid residues in a consistent background. It is important to employ channel proteins having a suitable genetic background when evaluating one or more mutations of interest so that the actual effect of the mutations is noted. In addition, when studying possible direct and indirect drug interactions with cardiac channels, it is important to assess those interactions in the proper context. The full import of the genetic background of mutations in $hNa_v1.5$ has not heretofore been understood.

BRIEF SUMMARY OF THE INVENTION

[0012] The present invention relates to novel $hNa_v1.5$ sodium channel α subunit polypeptides designated as SCN5A variants [H558;Q1077], [H558R], [Q1077del] and [H558R;Q1077del], as well as polynucleotides that encode the polypeptides. The novel $hNa_v1.5$ polypeptides differ from

the previously reported hH1, hH1a and hH1b sequences at amino acid positions 559, 618 and 1027.

[0013] The present invention is of particular interest in that the art has heretofore presumed that hH1, hH1a or hH1b encoded the "wild-type" or "reference" background sequence against which the effects of various *SCN5A* mutations should be judged. It is disclosed herein, however, that the disclosed sequences are observed in hundreds of individuals tested, establishing that they are the true standard background sequences in humans. The impact of selecting a proper background sequence when evaluating cardiac sodium channels is demonstrated herein in the Example below by showing the differential effect of a single amino acid change placed upon the background peptides of the invention as opposed to the same change placed upon the prior $hNa_v1.5$ peptides.

[0014] The present invention also includes various related nucleic acid molecules and polypeptides that are useful in various applications such as detecting the subunit and generating antibodies to the subunit. The present invention also relates to cloning and expression vectors and cells containing same. In addition, the present invention includes methods for screening for an agent for altering (increasing or reducing) sodium channel activities. Furthermore, methods of using the nucleic acids and polypeptides to detect the SCN5A variants disclosed herein and to generate antibodies to detect and purify the variants are also included in the present invention. New diagnostic and treatment strategies for various sodium channel-related diseases and conditions are also enabled by the present invention. The polypeptides of the SCN5A variants find particular application for use as a background into which putative disease causing mutations are introduced for functional analyses, where the background is more representative of the population than prior human cardiac sodium channels, particularly more representative than hH1, which has been considered a *de facto* standard channel, even though the inventors have determined that it is not widely distributed in the population at large.

[0015] It is an object of the present invention to identify some of the most common forms of a human cardiac sodium channel.

[0016] It is another object of the present invention to provide new tools for designing diagnostic and treatment strategies for sodium channel related diseases and conditions.

[0017] It is an advantage of the present invention that the SCN5A amino acid sequences are common in the human population.

[0018] It is a feature of the present invention that an SCN5A polypeptide of the invention has a threonine at amino acid position 559, a leucine at amino acid position 618, an arginine at amino

acid position 1027 and a histidine or arginine at amino acid position 558, and either has a glutamine at amino acid position 1077 or misses the glutamine at the 1077 position due to alternative mRNA splicing. Examples of the polypeptides of the present invention include but are not limited to SEQ ID NO:2, 4, 6 and 8. It is further understood by the inventors that the polypeptides of the invention represent suitable background protein sequences upon which one or more further mutations can be introduced using standard methods known in the art and that use of a polypeptide comprising 20 or fewer amino acid differences from SEQ ID NO:2, 4, 6 or 8 over the length of SEQ ID NO:2, 4, 6 or 8, is within the scope of the invention. More preferably the altered polypeptide comprises 10 or fewer differences, or 5 or fewer, and most preferably a single difference, from SEQ ID NO:2, 4, 6 or 8.

[0019] An additional set of polypeptides of the invention further comprise a leucine at position 1766, but otherwise retain the amino acid sequences of the aforementioned set of polypeptides.

[0020] In a related aspect, the invention also relates to an isolated polynucleotide that encodes any of the aforementioned polypeptides of the invention. A polynucleotide of the invention can be an isolated nucleic acid molecule such as an mRNA molecule, a single or double stranded DNA molecule or a cDNA molecule, whether or not provided on a cloning vector or expression vector, as well as the complement of any of the foregoing. If the polynucleotide is a nucleic acid molecule provided on an expression vector, it can contain such upstream and/or downstream regulatory elements as are needed to support transcription and translation of the polynucleotide of the invention.

[0021] Other objects, advantages and features of the present invention will become apparent from the following specifications and claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0022] Fig. 1 shows alternative splicing of *SCN5A* at Exon 18. The top panel shows an example of sequencing data of RT-PCR products from mRNA isolated from human ventricle (see Example below). Note the single sequence present in the Exon 17 coding region, but a dual sequence in the exon 18 coding region. The splice acceptor site in the genomic DNA has two "cag" repeats leading to the alternative splicing of a glutamine at position 1077. This results in two splice variants, one of 2015 amino acids (mRNA top) and one with 2016 amino acids (mRNA bottom).

[0023] Fig. 2 shows quantitative analysis of alternatively spliced variant *SCN5A* transcripts that either encodes Q1077 or excludes Q1077 (Q1077del). (A) Examples of RT-PCR products from 3 control experiments and 4 subjects. A sample without DNA (NEG) served as a negative control, and synthesized template of 135 bp containing the "cag" repeat (Q1077) and template of 132 bp without the "cag" repeat (Q1077del) served as positive controls. RT-PCR from samples of mRNA taken at autopsy from a subject with sudden infant death syndrome (SIDS), an infant without structural heart disease (Infant), an adult with no structural heart disease (Adult), and from a myomectomy specimen from a patient with hypertrophic cardiomyopathy (HCM) all show the presence of both transcripts. (B) Summary data from 20 subjects (five in each group) show that the transcript coding for Q1077del was consistently more abundant relative to Q1077. Bars represent the mean and standard deviation of the relative abundance of each transcript from 5 subjects determined by autoradiography phosphoimaging.

[0024] Fig. 3 shows voltage clamp data for SCN5A variants. Current traces for hH1 and four common SCN5A channel variants sequences are shown. Currents were elicited by step depolarizations from a holding potential of -140 mV to various test potentials of 24 ms duration from -140 mV to +60 mV. No obvious differences in current time course were noted

[0025] Fig. 4 shows activation, inactivation and recovery kinetics of SCN5 variants. (A) Summary data of peak current voltage relationships for currents obtained as in Fig. 3. Data were normalized to the peak I_{Na} in each data set. The lines shown were generated by a Boltzmann function $Gna = [1 + \exp (V_{1/2}-V)/\kappa]^{-1}$, where $V_{1/2}$ and κ are the mid-point and the slope factor, respectively, and $Gna = I_{Na}/(V-Vrev)$ where Vrev is the reversal potential. (B) Summary data for the voltage dependence of "steady-state" inactivation. I_{Na} obtained in response to a test depolarization to 0 mV from a holding potential of -140 mV, following 1 sec conditioning step to the various conditioning potentials (Vc). In order to normalize the capacity transients a 0.2 ms step back to -140 was applied before a test depolarization. The voltage dependent availability from inactivation relationship was determined by fitting the data to the Boltzmann function: I_{Na} $I_{Na\text{-}max}\,[1+ \exp (Vc-V_{1/2})/\kappa]^{-1}$, where the $V_{1/2}$ and κ are the midpoint and the slope factor, respectively, and V is the membrane potential. (C) Summary data for recovery from inactivation. Recovery from inactivation was assessed using a two pulse protocol where a conditioning step of 1 sec to 0 mV inactivated I_{Na} followed by a test pulse to 0 mV after a recovery period "t" at a recovery potential of -140 mV. The peak I_{Na} in response to the test pulse was normalized to the maximum peak current and plotted versus "t." This recovery process was fit to the sum of two exponentials: Normalized $I_{Na}\,[A_f \exp (-t/\tau_f)] +[A_s \exp (-t/\tau_s)]$ where t is the recovery time interval,

τ_f and τ_s are the fast and slow time constant, and A_f and A_s are the fraction of the recovery components.

[0026] Fig. 5 shows summary data for peak I_{Na} densities for the variants shown. Currents were elicited by a step depolarization to -20 mV from a holding potential of -140 mV and normalized to membrane capacitance. Data for the hHl clone is included as an historical control. Results for [H558R] co-expressed with the β1 subunit are shown in the rightmost bar. The bars depict the mean and standard error of the mean derived from n measurements with the n for each construct shown within the bar. One way ANOVA (Deg. of freedom 124) with Bonferroni t-test was used to assess significance of differences in these amplitudes. By ANOVA, the I_{Na} density for [H558R] was significantly lower ($p < 0.05$) than [Q1077del] and [H558R;Q1077del] but not the Q1077 containing variants hH1 or [H558;Q1077].

[0027] Fig. 6 shows that [H558R] has normal trafficking to the cell surface. Standard light photography in the left-most image of each panel is compared with confocal micrographs of human embryonic kidney (HEK) cells with immunostaining against a FLAG epitope inserted into the Na channel. (A) Non-transfected HEK-293 cell shows no immunostaining. (B) The [Q1077del] channel shows normal immunostaining pattern in the periphery and around the nucleus. (C) The [H558R] also shows normal staining pattern indicating that it trafficks to the cell surface. (D) With the mutation M1766L in the [Q1077del] the second image shows immunostaining is confined to a perinuclear location. The third image shows immunostaining with an endoplasmic reticulum marker (see Example below) and the fourth image (where the previous two images are superimposed) shows colocalization of the non-trafficking channel with the endoplasmic reticulum marker. (E) When the M1766L mutation was engineered into the [H558R;Q1077] background, normal trafficking to the cell periphery was seen. All results represented here were seen in at least 7 additional experiments.

DETAILED DESCRIPTION OF THE INVENTION

[0028] It is disclosed here that none of the existing *SCN5A* clones (hH1, hH1a and hH1b) represents a common sequence for *SCN5A* because each contains a rare variant at one or more of amino acid positions 559, 618 and 1027 (see Table 1). The inventors have identified four groups of SCN5A variants that represent the most common SCN5A variants with regard to the above amino acid positions as well as two additional positions, 558 and 1077. The *SCN5A* background can be important for the function of disease causing mutations. For example, the Brugada

syndrome mutation T1620M showed a trafficking defect only with the relatively uncommon variant R1232W in the background (Baroudi, G. et al., *Circulation Research* 90:E11-E16, 2002). In another example, the H558R background affected the kinetics of the conduction disease mutation T512I (Viswanathan, PC et al., *J. Clin. Invest.* 111:341-346, 2003). In the Example below, M1766L arrhythmia mutation affected SCN5A trafficking only in the [1077del] background but not [H558R;1077del] background. Thus, it is important to study and test disease-causing or potentially disease-causing mutations in a relevant *SCN5A* background. It is also important to use a relevant *SCN5A* background for drug screening. Information on *SCN5A* background of a patient can be important for diagnostic and therapeutic purposes. The disclosure of the most common SCN5A variants here provides new tools to conduct SCN5A-related tests.

[0029] All four groups of SCN5A variants identified by the inventors have a threonine at amino acid position 559, a leucine at amino acid position 618 and an arginine at amino acid position 1027. However, they differ at amino acid positions 558 and 1077. Group 1 has a histidine at amino acid position 558 and a glutamine at amino acid position 1077. Group 2 has an arginine at amino acid position 558 and a glutamine at amino acid position 1077. Group 3 has a histidine at amino acid position 558 with the glutamine at amino acid position 1077 deleted. Group 4 has an arginine at amino acid position 558 with the glutamine at amino acid position 1077 deleted. Other positions of the SCN5A variants in these four groups can vary. For example, amino acid position 1103 for all groups can either be a serine (majority of the overall population) or a tyrosine (13.2% of the black population). The expected overall population frequencies for variant groups 1-4 are 24.5%, 10.5%, 45.5% and 19.5%, respectively. An example of an SCN5A amino acid sequence for each of the variant groups 1-4 is provided as SEQ ID NO: 2, 4, 6 and 8, respectively. The corresponding nucleotide sequences are SEQ ID NO:1, 3, 5 and 7, respectively.

[0030] The presence or absence of a glutamine at position 1077 is believed to arise from alternative mRNA splicing as part of the normal protein expression process. An individual can be homozygous or heterozygous for histidine or arginine at position 558. Individuals who are heterozygous at position 558 would, therefore, be expected to have all four forms of the $hNa_v1.5$ ion channel. Position 558 is believed to be located at or near a site of protein kinase A (PKA) phosphorylation in $hNa_v1.5$ sodium ion channels, and this amino acid position may play a role in regulation of the channel function.

[0031] For purpose of the present invention, the amino acids of the SCN5A variants are numbered by referring to the 2016 amino acid sequence GenBank Accession No. AC137578. Thus, in a

variant with a 1077 deletion, the amino acid after amino acid 1076 is numbered 1078 so that the last amino acid is still 2016 instead of 2015. For example, the recently described polymorphism that appears to predispose to ventricular ectopy would be referred to as S1103Y (Chen, S. et al., *J. Med. Genet.* 39:913-915, 2002) rather than "Y1102" (Viswanathan, PC et al., *J. Clin. Invest.* 111:341-346, 2003). Channels with sequence variations from the reference sequence are denoted by the amino acid substitutions separated by semicolons and contained in brackets. Accordingly, SCN5A variant groups 1-4 as disclosed here are referred to as [H558;Q1077], [H558R], [Q1077del], and [H558R;Q1077del], respectively. It is noted that in the attached sequence listing, the amino acids in SEQ ID NO:6 and 8 are numbered under the 2015 system because the PatentIn program automatically numbers the amino acids consecutively.

[0032] The term "isolated nucleic acid" or "isolated polypeptide" used in the specification and claims of the present invention means a nucleic acid or polypeptide isolated from its natural environment or prepared using synthetic methods such as those known to one of ordinary skill in the art. Complete purification is not required in either case. Amino acid and nucleotide sequences that flank a polypeptide or polynucleotide that occurs in nature, respectively, can but need not be absent from the isolated form. The polypeptides and nucleic acids of the invention can be isolated and purified from normally associated material in conventional ways such that in the purified preparation the polypeptide or nucleic acid is the predominant species in the preparation. At the very least, the degree of purification is such that the extraneous material in the preparation does not interfere with use of the polypeptide or nucleic acid of the invention in the manner disclosed herein. The polypeptide or nucleic acid is preferably at least about 85% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

[0033] Further, an isolated nucleic acid has a structure that is not identical to that of any naturally occurring polynucleotide or to that of any fragment of a naturally occurring genomic polynucleotide spanning more than three separate genes. An isolated nucleic acid also includes, without limitation, (a) a polynucleotide having a sequence of a naturally occurring genomic or extrachromosomal nucleic acid molecule but which is not flanked by the coding sequences that flank the sequence in its natural position; (b) a polynucleotide incorporated into a vector or into a prokaryote or eukaryote genome such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are polynucleotides present in mixtures of

clones, e.g., as these occur in a DNA library such as a cDNA or genomic DNA library. An isolated nucleic acid can be modified or unmodified DNA or RNA, whether fully or partially single-stranded or double-stranded or even triple-stranded. A nucleic acid can be chemically or enzymatically modified and can include so-called non-standard bases such as inosine.

[0034] In one aspect, the present invention relates to an isolated polypeptide comprising the amino acid sequence of an SCN5A variant disclosed herein. The SCN5A variant can be any SCN5A sequence that has a threonine at amino acid position 559, a leucine at amino acid position 618, an arginine at amino acid position 1027, a histidine or arginine at amino acid position 558, and a glutamine or glutamine deletion at amino acid position 1077. Examples amino acid sequences of an SCN5A variant are provided as SEQ ID NO:2, 4, 6 and 8. Generally speaking, an SCN5A variant of the present invention will not differ from SEQ ID NO:2, 4, 6 or 8 at more than 20 amino acid positions other than positions 558, 559, 618, 1027 and 1077. Preferably, the difference is limited to 10 or fewer, more preferably 5 or fewer, and most preferably 1 or fewer positions. However, it is understood that substitutions such as a conservative substitution can be introduced into non-critical amino acid positions and this will not materially affect the function even when more than 20 amino acids are substituted. An SCN5A variant with such substitutions is within the scope of the present invention.

[0035] Furthermore, an isolated polypeptide of the invention can also include one or more amino acids at either or both of the N-terminus and C-terminus of an SCN5A variant disclosed herein, where the additional amino acid(s) do not materially affect the function of the variant, which can be determined using the parameters shown in Table 3. Any additional amino acids can, but need not, have advantageous use in purifying, detecting, or stabilizing the polypeptide. Likewise, small deletions or other rearrangements in the polypeptide that do not affect the function of the polypeptide are also within the scope of the invention. Such deletions are preferably deletions of fewer than 100 amino acids, more preferably of fewer than 50 amino acids, still more preferably of fewer than 10 amino acids.

[0036] In a related aspect, the present invention also includes an immunogenic fragment of an SCN5A variant disclosed herein and an antibody that binds specifically to such an immunogenic fragment. Such immunogenic fragments are used to generate specific antibodies that can be used to detect or isolate an SCN5A variant of the present invention, or both. In general, the immunogenic fragments contain at least 15 continuous amino acids, preferably at least 20 continuous amino acids, and most preferably at least 25 continuous amino acids of an SCN5A

variant in which the continuous amino acids include position 558. An antibody that is specific for a variant of the present invention will have a higher affinity for the variant than for hH1, hH1a, or hH1b. It is well within the ability of a skilled artisan to make monoclonal or polyclonal antibodies against some or all of the polypeptides and to assess the specificity of the antibodies. Furthermore, as it is now shown by the inventors that the variants disclosed herein are the "common" or "standard" forms of the $hNa_v1.5$ protein, an antibody that merely identifies the protein may be sufficient for various uses, without regard to its specificity relative to hH1, hH1a and hH1b.

[0037] In another aspect, the present invention relates to an isolated nucleic acid containing a coding polynucleotide or its complement wherein the coding polynucleotide has an uninterrupted sequence that encodes a polypeptide of the invention as set forth above. A nucleic acid containing a polynucleotide that is at least 80% identical to the coding polynucleotide or its complement over the entire length of the coding polynucleotide can be used as a probe for detecting the coding polynucleotide and is thus within the scope of the present invention.

[0038] In a related aspect, any nucleic acid of the present invention described above can be provided in a vector in a manner known to those skilled in the art. The vector can be a cloning vector or an expression vector. In an expression vector, the polypeptide-encoding polynucleotide is under the transcriptional control of one or more non-native expression control sequences which can include a promoter not natively found adjacent to the polynucleotide such that the encoded polypeptide can be produced when the vector is provided in a compatible host cell or in a cell-free transcription and translation system. Such cell-based and cell-free systems are well known to the skilled artisan. Cells comprising a vector containing a nucleic acid of the invention are themselves within the scope of the present invention. Also within the scope of the present invention is a host cell having the nucleic acid of the present invention integrated into its genome at a non-native site. Further, the above cells of the present invention can contain *SCN5A* variants from more than one of the four variant groups.

[0039] The present invention also includes an isolated nucleic acid molecule that contains a fragment of at least 12, 15, 20 or 25 contiguous nucleotides of an *SCN5A* variant disclosed herein, or its complement, particularly a fragment that comprises codon 558, 1077, or both. Such a nucleic acid molecule can be used to detect the expression of the *SCN5A* variant in a cell. The detection reaction can be run under stringent hybridization conditions, for example, by hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at

room temperature. Moderately stringent conditions which include washing in 3x SSC at 42°C can also be used.

[0040] The present invention also enables a screening method for agents that can either inhibit or enhance sodium channel activities. In such a method, an agent is exposed to a host cell of the present invention that expresses one or more *SCN5A* variants disclosed herein and the agent's effect on the variants' activities is determined by comparing to control cells that are not exposed to the agent. The activity of an SCN5A variant can be measured in many ways, including but not limited to measuring a sodium current across the cell membrane, a sodium current kinetic activity, a membrane potential, or an intracellular sodium level. Agents that can modulate the expression of an *SCN5A* variant can be screened similarly using cells which contain the variant whose expression is controlled by the native expression control sequences. Also, a phenotype associated with over-expression of a sodium channel or absence of expression (e.g., in a transgenic or knockout animal) can be monitored. *In vitro*, an effect on action potential can be measured after a channel of interest is transfected into suitable cells, such as cardiac cells. An arsenal of agents affecting the sodium channel activity is desired because many diseases and conditions, such as arrhythmias and Brugada syndrome, result from elevated or reduced sodium channel activity. Particularly in view of the understanding that various forms of the sodium channel α subunit differ functionally, it is important to evaluate the effects of every form that may be present in an individual. Indeed, one can tailor a suitable treatment to an individual after evaluating the form of α subunit present in that individual. Sodium channel activity means the open channel activity leading to a peak sodium current. Sodium channel activity is enhanced or inhibited when the open state probability is greater or less, and the peak current is higher or lower, respectively, than in the absence of a modulating agent.

[0041] The human embryonic kidney cell line (HEK) described in the Example below is a suitable cell line that can be transfected with various *SCN5A* constructs of the present invention to screen for agents that can affect the function of an SCN5A variant. This cell advantageously lacks endogenous SCN5A proteins to interfere with the signal from a transfected *SCN5A* of interest. However, other suitable cells can also be used. If a cell, such as a heart cell, which expresses endogenous *SCN5A* is used, the signal attributable to the endogenous protein must be subtracted when the activity of a transfected *SCN5A* is measured.

[0042] Batteries of agents for screening are commercially available in the form of various chemical libraries including peptide libraries. Examples of such libraries include those from

ASINEX (e.g., the Combined Wisdom Library of 24,000 manually synthesized organic molecules) and from CHEMBRIDGE CORPORATION (e.g., the DIVERSet™ library of 50,000 manually synthesized chemical compounds; the SCREEN-Set™ library of 24,000 manually synthesized chemical compounds; the CNS-Set™ library of 11,000 compounds; the Cherry-Pick™ library of up to 300,000 compounds). Once an agent having desired ability to increase or decrease activity of the sodium channel protein is identified, further iterations of the screen using one or more libraries of derivatives of that agent can be screened to identify agents having superior effects.

[0043] The above screening methods also enable one to determine the likelihood that an agent intended to be administered to a human or non-human subject will induce an undesired and unintended side effect, namely by altering the activity of the cellular SCN5A in the subject.

[0044] The present invention also enables a skilled artisan to determine whether a mutation is associated with a sodium channel-related disease on a common *SCN5A* background. To do this, a mutation is introduced into an *SCN5A* variant disclosed herein and the effect of the mutation is then tested in a suitable model for the disease.

[0045] The polypeptides, polynucleotides and antibodies of the invention find particular utility as screening tools for identifying to which of the four SCN5A groups a particular subject belongs. This information is useful in several aspects. For example, it may help assess the subject's predisposition to acquired arrythmias. For instance, if a subject has a high proportion of low-expressing channels such as [H558R], the subject could be predisposed to develop acquired arrythmias. Also, for a subject that suffers from a sodium channel-related disease, knowing the SCN5A background of the subject can help choose treatment strategies.

[0046] With the disclosure herein, it is well within a skilled artisan's ability to determine to which of the four SCN5A groups a subject belongs. Such determination can be made at the polynucleotide level or protein level. At the polynucleotide level, primers and probes that specifically amplify or hybridize to each of the four SCN5A groups can be used. Alternatively, direct sequencing can also be used. At the protein level, antibodies specific for each of the four SCN5A groups can be developed and used. Alternatively, the amino acid sequence of an SCN5A protein can be determined directly.

[0047] Any product of the invention described herein can be combined with one or more other reagent, buffer or the like in the form of a kit useful, e.g., for diagnostic or therapeutic purposes, in accord with the understanding of a skilled artisan.

[0048] The present invention is not intended to be limited to the foregoing, but rather to encompass all such variations and modifications as come within the scope of the appended claims. The invention will be more fully understood upon consideration of the following Examples which are, likewise, not intended to limit the scope of the invention.

Example

Materials and Methods

[0049] *Genotyping for residues 558, 559, 618 and 1027*: Allele frequencies for the *SCN5A* variants (H558R in exon 12, T559A in exon 12, L618I in exon 12, and R1027Q in exon 17) were established by direct genomic DNA sequencing of 400 reference alleles derived from the 100 Caucasian human variation panel and the 100 African-American human variation panel (Coriell Cell Repositories and the National Institute of General Medical Sciences). Protein-encoding sequences harboring these variants were amplified by polymerase chain reaction (PCR) using previously published intron/exon based primers 5 and subsequently sequenced using automated dye-terminator cycle sequencing on an ABI Prism 377. The fluorescent detection stems from reporter dyes bound to the ddNTP terminator nucleotides. The Big-Dye Terminator Cycle Sequencing v 1.0 kit from Applied Biosystems (ABI part# 4303154) was used for sequencing of all samples. To determine the status at residues 558, 559, and 618, *SCN5A* exon 12 was analyzed by PCR amplification of two overlapping fragments 12A (forward primer - GCCAGTGGCTCAAAAGACAGGCT (SEQ ID NO:9) and reverse primer – CCTGGGCACTGGTCCGGCGCA (SEQ ID NO:10)) and 12B (forward primer - CACCACACATCACTGCTGGTGC (SEQ ID NO:11) and reverse primer – GGAACTGCTGATCAGTTTGGGAGA (SEQ ID NO:12)). PCR amplification reactions for amplicons 12A and 12B were performed in 20 μL volumes using 50 ng of genomic DNA, 16 pmol of each primer, 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, and 1.0 U of Amplitaq Gold (Applied Biosystems, Branchburg, NJ). The reaction mixture was subjected to a 95°C initial denaturation for 5 min, followed by 5 cycles of 94°C for 20 s, 64°C for 20 s, and 72°C for 30 s; then an additional 35 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 30 s, and a final extension of 72°C for 10 min. PCR reactions used to amplify *SCN5A* exon 17 (R1027Q variant) were performed in 20 μL volumes using 50 ng of genomic DNA, 16 pmol of each primer (forward primer - GCCCAGGGCCAGCTGCCCAGCT (SEQ ID NO:13) and reverse primer -CTGTATATGTAGGTGCCTTATACATG (SEQ ID NO:14)), 200 pM of

each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.0 mM $MgC1_2$, 8% DMSO and 1.0 U of Amplitaq Gold (Perkin-Elmer). The cycling conditions were as follows: 94°C initial denaturation for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. PCR products for exon 12 (12A and 12B) and exon 17 were enzymatically treated to remove unincorporated dNTP and primers with EXOSAP-it (USB Inc., Cleveland, OH) following the manufacturer's protocol. Treated products were sequenced using dye-terminator cycle sequencing with an ABI 377, and the resulting chromatograms were analyzed for the specific variant.

[0050] *Identification, characterization, and quantification of alternatively-spliced SCN5A transcripts encoding for either insertion or deletion of glutamine (Q) at residue 1077*: Direct DNA sequencing was performed on exon 17/18 targeted RT-PCR generated products derived from messenger RNA that was extracted from myocardial tissue obtained at either i) autopsy of sudden infant death syndrome (n=5), non-accidental infant death (n=5), or accidental adult death victims (n=5) or ii) surgical myectomy in adults with hypertrophic cardiomyopathy (n=5). Total RNA from an approximate 25 mg piece of heart tissue was isolated using the Rneasy™ Fibrous Tissue Mini kit (Qiagen, Valencia, CA), and first-strand cDNA synthesis was performed in triplicate on 500 ng of total RNA using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA) following the manufacturer's specifications. PCR was performed in 20 μL volumes using 2 μL of cDNA, 16 pmol of each primer (17F forward: CCAAGAAGAGGATGAGGAGA (SEQ ID NO:15),18R reverse: GAGGCAGTCGCTGACACC (SEQ ID NO:16)), 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgC1_2$, and 1.0 U Amplitaq Gold (Perkin-Elmer). The cycling conditions were as follows: 94°C initial denaturation for 10 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. PCR products were purified with EXOSAP-it (USB Inc., Clevland, OH) and sequenced using an automated AB1377 sequencer (Applied Biosystems, Inc., Foster City, CA). The relative quantity of the 2 alternatively-spliced transcripts was then quantified. Triplicate cDNA samples from each case were subject to PCR amplification using the same PCR primers and conditions as above, with the addition of 0.2 μl of alpha-^{32}P dCTP (10 mCi/ml). Since all samples were shown to harbor both transcripts by direct DNA sequencing, control templates representing homozygous Q1077 and Q1077del transcripts were synthesized and PAGE purified by Integrated DNA technologies, Inc. (Coralville, IA). The resulting radionucleotide incorporated PCR products representing 1) the transcript (135 base pairs) encoding the insertion of glutamine at residue 1077 (Q1077) or 2) the transcript (132 base pairs) that does not encode for glutamine at residue 1077 (Q 1077del) were

separated by denaturing gel electrophoresis at 70 watts for 2 hrs and 45 min on a 6% polyacrylamide (19:1), 7 M urea gel. Autoradiography with a phosphoimager 445 SI and ImageQuant v 5.0 software (Molecular Dynamics, Piscataway, NJ) were used for signal quantification of the two alternatively-spliced transcripts.

[0051] *Gene expression, mutagenesis, and nomenclature*: The *SCN5A* clone hHl (GenBank No. M77235) was kindly provided by Dr. Al George (Gellens, ME et al., *Proc. Natl. Acad. Sci. U.S.A* 89:554-558, 1992, which is herein incorporated by reference in its entirety) in prcCMV (Invitrogen). The hHlc construct (GenBank No. AY148488) was made from the hHlb clone (GenBank AF482988) (Ye, B. et al., *Physiol. Genomics* 12:187-193, 2003, which is herein incorporated by reference in its entirety) by mutating the arginine (R) at 558 to histidine (H), and the isoleucine (I) at positions 618 to leucine (L) by methods described below. The consensus reference sequence (GenBank No. NM_000335 June 2003) has 2015 amino acids and is identical to hHlc. For naming and reference purposes we prefer the full-length 2016 amino acid deduced sequence from the IHGSC (GenBank No. AC137587, deposited April 2003) as the base sequence for *SCN5A* because this nomenclature retains the well-established numbering system based upon the 2016 amino acids encoded by the original *SCN5A* clone hHl (Gellens, ME et al., *Proc. Natl. Acad. Sci. U.S.A* 89:554-558, 1992). Channels with sequence variations from the reference sequence are denoted by the amino acid substitutions separated by semicolons and contained in brackets as recommended by den Dunnen and Antonarakis (den Dunnen, JT et al., *Hum. Mutat.* 15:7-12, 2000). The variant channels [H558R], [Q1077del], [H558R;Q1077del], [H558R;Q1077del;M1766L], and [Ql077del;Ml766L] were made by mutagenesis at appropriate residues by the following method.

[0052] Mutations were generated using Excite® mutagenesis kit (Stratagene, La Jolla, CA) using the protocol suggested by the manufacturer. DNA was isolated and purified with the Qiagen (Valencia, CA) column and protocol. All constructs were sequenced to verify incorporation of the intended amino acid change and to confirm that no unwanted changes were introduced. These constructs were placed in pcDNA3 (Invitrogen, Carlsbad, CA) and expressed in HEK293 cell line by transfection with 1.5 μg of plasmid DNA using Superfect (Qiagen) according to the protocol recommended by the manufacturer. A GFP protein was co-transfected (at 1:10) as a marker to identify the transfected cells. HEK 293 cells were harvested 24 hours after transfection to measure macroscopic current as previously described (Nagatomo, T. et al., *Am. J. Physiol. (Heart 44)* 275:H2016-H2024, 1998, incorporated by reference in its entirety). Experiments were done with transient transfection unless otherwise noted. A few experiments used cell lines expressing

these variants in a stable manner. For these stable cell lines, pcDNA3 (Invitrogen) plasmid DNA containing the hNav1.5 α subunit was transfected into HEK-293 cells and selected as follows. Approximately 1 x 10^5 cells were plated on a 60 mm diameter plate (Falcon 3001) approximately 24 hours prior to transfection in 3 mL MEM-complete media (Minimal Essential Media (Gibco/Invitrogen) supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum, 1 mM sodium pyruvate solution, 0.1 mM non-essential amino acids, 10,000 U of Penicillin and 10,000 mg of streptomycin). 1.5 μg of plasmid DNA was mixed along with 10 μL of Superfect reagent (Qiagen) into 140 μL of Opti-MEM (Gibco/Invitrogen) and allowed to incubate at room temperature for 10 min to allow for the DNA to bind to the Superfect. The HEK cells were then incubated with this DNA and Superfect solution in 1 mL MEM-complete for 3.5 hrs, at which point the media was replaced with 3 mL of MEM-complete. 24 hours post-transfection 800 ng/mL of G418 antibiotic was added. MEM-complete + G418 medium was thereafter replaced every 72 to 96 hours. After 3-4 weeks, single colonies were isolated from the transfected plate and grown in separate wells of a 6-well plate (Costar 3516, Corniing, NY). RNA was isolated (RNAisol from LPS, Moonachie, NJ) and screened by RT-PCR analysis. Colonies that tested RT-PCR-positive were then analyzed by voltage clamp.

[0053] *Voltage-clamp techniques*: The whole cell patch-clamp technique was utilized to measure macroscopic I_{Na} (Nagatomo, T. et al., *Am. J. Physiol. (Heart 44)* 275:H2016-H2024, 1998). The pipette solution contained 120 mM CsF, 15 mM CsCl, 2 mM EGTA, 5 mM HEPES and 5 mM NaCl (pH7.4 with CsOH). Data were recorded at room temperature using pCLAMP 8 (Axon Instruments). The voltage-clamp protocols are described briefly with the data and have been published previously in detail (Valdivia, CR et al., *J. Mol. Cell Cardiol.* 34:1029-1039, 2002). Peak I_{Na} and late I_{Na} were obtained after passive leak subtraction as described previously (Nagatomo, T. et al., *Am. J. Physiol. (Heart 44)* 275:H2016-H2024, 1998). Parameter fits were obtained using Clampfit 8 (Axon Instruments). One way ANOVA was performed to determine statistical significance among 3 or more groups of mean data. Statistical significance was determined by a P value < 0.05.

[0054] *Immunocytochemistry*: The FLAG epitope was introduced between S1 and S2 in domain I for channels used in the immunocytochemistry experiments. Transfected and non-transfected HEK293 cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes. The fixed cells were blocked with 5% goat serum and 0.2% Triton PBS solution at room temperature for 30 minutes. After the blocking procedure, the cells were incubated with the mouse anti-FLAG M2 primary antibody (Stratagene®, La Jolla, CA) at the ratio of 1:2000 overnight at 4 °C. On the

next day, the cells were washed with PBS before 1:100 fluorescein-conjugated goat anti-mouse antibody (Jackson, West Grove, PA) was applied as the secondary antibody and allowed to react for 1 hour at room temperature in the dark. The rabbit anti-Calnexin IgG was used as endoplasmic reticulum (ER) marker to test for co-localization. A 1:1000 dilution of the rabbit anti-Calnexin IgG in 250 μl was applied to transfected cells immediately after incubation with fluorescein-conjugated goat anti-mouse secondary antibody. The reaction was incubated for 2 hrs at 37°C. After incubating with anti-Calnexin IgG, the cells were washed twice with 300 μl of PBS and incubated with 150 μl of Texas Red-conjugated goat anti-rabbit secondary antibody (Jackson, West Grove, PA) at the ratio of 1:100. The incubation with Texas Red-conjugated goat anti-rabbit 2nd antibody was done at room temperature in the dark for 1 hour. The cells were washed with PBS solution and fixed with a solution containing 90% glycerol and 10% Na carbonate. A Bio-Rad MRC 1024 Laser Scanning system with 15 mW mixed gas (Krypton/Argon) laser was utilized to view immunofluorescently labeled cells. The Bio-Rad MRC 1024 system was mounted on a Nikon Diaphot 200 inverted microscope. Images of the fluorescent-labeled cells were scanned under 40x objective and 2x zoom. The confocal system was set to 3.6 for iris, laser power at 100% and camera sensitivity gain to 900. A Kalman collection filter with 5 frames per image was applied to record the image.

Results

[0055] *Sequence comparisons for SCN5A clones and genotyping of control panels*: The sequences of hH1 (Gellens, ME et al., *Proc. Natl. Acad. Sci. U.S.A* 89:554-558, 1992), hHla (Hartmann, HA et al., *Circulation Research* 75:114-122, 1994, which is herein incorporated by reference in its entirety), and hHlb (Ye, B. et al., *Physiol. Genomics* 12:187-193, 2003) were compared and the amino acid differences at 5 residues are shown in Table 1 along with the GenBank Accession number where available. In a previous study of the hHlb clone (Ye, B. et al., *Physiol. Genomics* 12:187-193, 2003), hH1 and hH1a were re-sequenced and the differences between these two clones were found to be only the 3 residues shown in Table 1 rather than the 9 reported previously.

Table 1 – Comparisons for deduced SCN5A/$Na_v1.5$ sequences from full-length cDNA clones (hH1, hH1a, hH1b), genomic sequencing (hH1c) and genomic databases (Celera, IHGSC).

SCN5A Variant Group		1	2				3	4
Common[a] Name		**IHGSC**		**hH1**	**hH1a**	**hH1b**	**hH1c[d]**	
Accession No.[b]		AC137587	NA	M77235	NA	AF482988	AY148488	NA
AA[c] No.	558	R	R	H	H	R	H	R
	559	T	T	T	A	T	T	T
	618	L	L	L	L	I	L	L
	1027	R	R	Q	R	R	R	R
	1077	Q	Q	Q	Δ	Δ	Δ	Δ
Variant[e] name		[H558; Q1077]	[H558R]	[R1027Q]	[T559A; Q1077del]	[H558R; L618I; Q1077del]	[Q1077del]	[H558R; Q1077del]
Population[f] Frequency		24.5%	10.5%	0%	0%	0%	45.5%	19.5%

[a]IHGSC = International Human Genome Sequencing Consortium sequence and reference sequence; hH1, hH1a, hH1b = previously cDNA clones of SCN5A; hH1c = Common sequence from genomic sequencing in control subjects (a search of Celera human genome database identified the same sequence).

[b]Accession No. = GenBank Nucleotide accession numbers, where available.

[c]AA No. = Amino Acid residue number in the protein, using the full length numbering consistent with the IHGSC database as well as the originally isolated SCN5A. At all 2011 the positions not noted in the table above the amino acids are identical in all sequences. "Δ" indicates there is no amino acid present at this location in the amino acids (thus, the product is 2015 amino acids in length, rather than 2016). The amino acid frequencies in the population of all proteins in the population of all studied humans at positions 558, 559, 618, 1027 and 1077 are 70% H, 100% T, 100% L, 100% R and 65% Δ, respectively.

[d]A search of Celera human genome database identified the same sequence

[e]Variant name = name relative to [H558;Q1077] (defined herein as identical to IHGSC).

[f]Population Frequency = estimated percentage of protein products in the study population that have this sequence.

[0056] We investigated allelic frequencies from genomic DNA at the 5 positions in question from a panel of 200 human controls (100 white subjects and 100 black subjects). The most common residues at these 5 positions are reported as hHlc in Table 1. For positions 559, 618, and 1027, 100% of the 400 reference alleles showed T559, L618, and R1027, indicating that each of the existing clones contained a rare variant and that none represented the common sequence (Table 1). A search of the Celera human genome database showed that the deduced amino acid sequence agreed with hH1c. The deduced amino acid sequence in the NIH International Human Genome Sequencing Consortium (IHGSC) also agreed with the hHlc sequence. However, the IHGSC sequence contains the additional glutamine (Q) at position 1077 as found in the hH1 clone but not hH1a or hH1b.

[0057] *A common polymorphism, H558R*: Residue 558 hosts the common polymorphism involving a substitution of histidine (H) with arginine (R) H558R (Iwasa, H. et al., *J. Hum. Genet.* 45:182-183, 2000) with a reported frequency in the population of 19-24% (Yang, P. et al., *Circulation* 105:1943-1948, 2002). We confirm that H558R is a common polymorphism (Table 2) among both blacks and whites; the apparent higher incidence of H558R in blacks is not statistically significant. We also show the frequency of heterozygosity and homozygosity at this position (Table 2).

Table 2. Genotype and Allelic Frequency of H558R Polymorphism

Ethnicity	Sample Size	HH	HR	RR	H allele	R allele
White	100	65	30	5	0.8	0.2
Black	100	53	40	7	0.73	0.27

[0058] *A common alternatively-spliced variant, Q1077del*: The hH1 clone contained a glutamine (Q) residue at both amino acid positions 1076 (the final codon of exon 17) and 1077 (the first codon of exon 18) (Gellens, ME et al., *Proc. Natl. Acad. Sci. U.S.A* 89:554-558, 1992). The acceptor site sequence for exon 18 was annotated as ggggtcttttcagCAGGAATCC (SEQ ID NO:17) where the lower case letters represent the intronic sequences and the upper case letters represent the 5' exonic sequences of exon 18 (see Fig. 1). The underlined lower case letters indicate the predicted "ag" acceptor site rule for splice site recognition. However, splice-site analytical tools indicate that the CAG following the ag shown above could also comprise the terminal intronic sequence and may be the preferred acceptor site for splicing, resulting in a deletion of glutamine at residue 1077 (Q1077del).

[0059] To answer the question whether SCN5A most commonly has a glutamine (Q) at both amino acid positions 1076 and 1077 as in hH1 or only 1076 as in hH1a and hH1b, we performed direct sequencing on exon 17/18-targeted RT-PCR generated products derived from mRNA isolated from human left ventricular myocardial specimens from sudden infant death syndrome (SIDS), infants with structurally normal heart, adults with hypertrophic cardiomyopathy, and adults with structurally normal hearts. All subjects were heterozygous for a "cag" in-frame insertion indication the universal presence of alternative-splicing involving this acceptor site (Fig. 1). The relative abundance for each alternatively-spliced transcript was quantified by autoradiography and phosphoimaging (Fig. 2). Examples of RT-PCR products generated from myocardial RNA (Fig. 2A) show the expected size products for control experiments with the 135

bp template containing the extra cag codon (Q1077) and for the 132 bp template lacking the extra cag codon (Q1077del). Summary data (Fig. 2B) show that Q1077del was the preferred alternatively-spliced variant being significantly more abundant in every group tested. Overall, the proportion of alternatively-spliced variant containing Q1077 was 35 ± 2.0% (range 31-38%, n=20) and the Q1077del transcript was 65 ± 2.0% (range 62-69, n=20). The total test group contained 9 males and 11 females, and the infant group contained 5 white and 5 black subjects. This degree of preferential splicing was not influenced by age, sex, race, or presence of ventricular hypertrophy. In addition, these ratios of Q1077 and Q1077del-transcripts were maintained regardless of whether RNA was obtained from right atrium, left atrium, right ventricle, or left ventricle.

[0060] *Four common SCN5A variants in the human population*: Based upon the predicted amino acid frequency estimates from our genomic sequencing in controls and our measurement of the frequency of the splice variant Q1077del, we estimated the population frequency of the existing clones and other full-length sequences (Table 1). These estimates assume independence between the probability of the Q1077del splice variant (65%) and the genomic variant containing H558R (30%). Note that the three clones hHl, hHla, and hHlb used in previous studies are estimated to have a very low frequency in the population because of the presence of a rare variant in each. The most common variant [Q1077del] at 45.5% is identical to the hHlc sequence, the Celera sequence, and also to the NCBI Reference Sequence for *SCN5A* (GenBank Accession number NM_000335). The full-length reference sequence SCN5A ([H558;Q1077]) is actually less frequent than [Q1077del] at 24.5%. Only slightly less common are the variants with the H558R polymorphism designated as [H558R;Q1077del] at 19.5% and [H558R] at 10.5% (by our convention, the [H558R] variant contains Q1077).

[0061] *Functional characterization of SCN5A variants*: Constructs for expressing [H558;Q1077] and the other common variants [H558R], [Q1077del] and [H558R;Q1077del] were made and transfected individually into HEK cells for kinetic studies. For comparison, we expressed hHl contemporaneously under the same conditions with these variants because most previous studies in the literature have used hHl. Representative currents for the four variants and hHl are shown in Fig. 3. For those variants that expressed robust current, no significant differences were identified in i) the parameters of activation (Fig. 4A, Table 3), ii) current decay (Table 3), iii) recovery from inactivation (Fig. 4C, Table 3) and iv) late or persistent currents (Table 3). The midpoint of inactivation for hHl, however, was significantly negative to the two variants lacking Q1077 [Q1077del] and [H558R;Q1077del] (Fig. 4B and Table 3).

Table 3. Voltage-dependent Kinetic Parameters for $hNa_v1.5$ Channels

	hH1	[Q1077del]	[H558R;Q1077del]	[H558;Q1077]
Activation				
$V_{1/2}$ (mV)	-40 ± 2	-42 ± 1.2	-44 ± 2	-40 ± 2
Slope factor	5.5 ± 0.3	4.9 ± 0.1	5.1 ± 0.2	4.9 ± 0.3
N	5	22	23	13
Inactivation				
$V_{1/2}$ (mV)	-92 ± 2*	-85 ± 1.2	-84 ± 1.3	-85 ± 4
N	18	26	14	5
Recovery				
τ_f (ms)	3.0 ± 2	2.6 ± 0.3	2.8 ± 0.4	3.3 ± 1.6
τ_s (ms)	81 ± 77	34 ± 4.7	53 ± 14	71 ± 40
A_s	22 ± 0.1	22 ± 0.5	17 ± 2	26 ± 0.9
N	7	19	14	4
Decay (-30 mV)				
τ_f (ms)	1.8 ± 0.8	1.3 ± 0.4	1.6 ± 0.6	1.0 ± 0.2
τ_s (ms)	11.4 ± 9.6	4.7 ± 3	8.1 ± 8.8	4.0 ± 2.0
A_f	0.64 ± 0.3	0.56 ± 0.2	0.64 ± 0.2	0.7 ± 0.2
N	8	4	6	5
Late I_{Na} (-20 mV)				
% of peak I_{Na}	-	0.85 ± 0.3	0.43 ± 0.2	0.85 ± 0.3
N	-	10	7	9

The data shown here are the mean ± SEM from curve fitting to N experiments. Activation, inactivation, and recovery from inactivation parameters were obtained as in Fig. 3. For the decay of I_{Na} at -30 mV, the portion of the I_{Na} trace after 90% of peak was fit a sum of exponentials: $I_{Na}(t) = 1 - (Af * \exp -t/\tau_f + A_s * \exp -t/\tau_s) +$ offset where t is time, τ_f and τ_s represent the time constant of the fast and slow components, and A_f and A_s are amplitudes of fast and slow component, respectively. Late I_{Na} was obtained at 720 ms after the depolarization as described previously (Valdivia, CR et al., *J. Mol. Cell Cardiol.* 34:1029-1039, 2002). Using one-way ANOVA with Bonferroni T-test, the midpoint of inactivation for hH1 marked by an asterisk was significantly negative to the variants lacking Q [H558R;Q1077del] and [Q1077del].

[0062] The most dramatic finding was that [H558R] which contains Q1077 expressed very low current density. Data for current density are summarized in Fig. 5 where the current density for [H558R] was dramatically and significantly lower than the other variants. Other constructs that contained Q1077 (hHl and [H558;Q1077]) also tended to have reduced currents but the difference did not reach statistical significance by ANOVA. RT-PCR of mRNA from cells with both transient and stable transfection of [H558R] showed abundant [H558R] transcript, but negligible currents. The β1 subunit increased current density when co-expressed with the α subunit of Nav1.5 (Nuss, HB et al., *J. Gen. Physiol.* 106:1171-1191, 1995). The β1 subunit also "rescued" the trafficking defective SCN5A mutations (Valdivia, CR et al., *Cardiovascular Research* 54:624-629, 2002). When [H558R] was co-expressed with the β1 subunit, increased currents were observed although not at the levels of the other channel α subunits expressed alone (Fig. 5). To

determine if the small currents seen with [H558R] could be endogenous currents, we voltage clamped untransfected HEK cells. In 8 cells, only 1 cell had 100 pA of current for an average density of less than 0.5 pA/pF compared with 2.8 pA/pF in [H558R] alone and 16 pA/pF in [H558R] co-transfected with the β1 subunit. We were unable to significantly increase endogenous currents in untransfected cells by co-transfection with β1 (1.0±0.5 pA/pF, n=9), incubation with mexiletine 100 μM for 24 hours (1.1±0.4 pA/pF, n=10), or incubation at 27°C (0.9±0.4 pA/pF, n=8). We conclude that [H558R] did indeed generate small currents. When both variants [H558R] and [H558R;Q1077del] were co-expressed (plasmid DNA 0.75 μg each), normal current densities were seen. We conclude that variants containing Q1077 tended to have lower I_{Na} density, and also tended to have the voltage-dependence of inactivation kinetics shifted in the negative direction. When the H558R polymorphism is expressed in the setting of the alternatively-spliced transcript that contains Q1077, then I_{Na} density was dramatically reduced.

[0063] *Cell trafficking of the [H558] variant*: Some SCN5A variants with decreased current density have been shown by immunocytochemistry to have defective trafficking (Ye, B. et al., *Physiol. Genomics* 12:187-193, 2003; Baroudi, G. et al., *Circulation Research* 90:E11-E16, 2002) and do not make it to the cell surface. To determine if [H558R] made it to the cell surface, we labeled the channel variants by inserting a FLAG epitope, and localized the channels by immunofluorescence and confocal microscopy. In each panel of Fig. 6 a light microscopy image is shown allowing for identification of the nucleus and the cell surface, followed by the confocal immunofluorescence image(s). A non-transfected cell gave no fluorescent signal as expected (Fig. 6A). The [Q 1077del] variant that gave robust current densities showed a rim of fluorescence at the cell surface as expected for a normally trafficking channel (Fig. 6B). The [H558R] variant expressed almost no current, but also showed fluorescence at the cell surface (Fig. 6C) suggesting that the lack of current was not caused by a trafficking defect.

[0064] As an example of a channel that is trafficking defective and as a positive non-trafficking control for our experiment with the [H558R] variant, we made the mutation M1766L in the variants and show data from two of these experiments (Fig. 6D & E). We had previously shown M1766L to be trafficking defective in hHla [T559A;Q1077del;M1766L] but to be normally trafficking in hHlb [H558R;L6181;Q1077del;M1766L] (Ye, B. et al., *Physiol. Genomics* 12:187-193, 2003). When the M1766L mutation was put into [Q1077del] to make [Q1077del;M1766L] (Fig. 6D), the fluorescence was restricted to the area around the nucleus without any labeling in the periphery, consistent with a trafficking defect. This eliminates the possibility that the rare variant T559A in hHla was responsible for the trafficking defect as previously described (Ye, B.

et al., *Physiol. Genomics* 12:187-193, 2003). An image with a marker for the endoplasmic reticulum is shown in the third panel of Fig. 6D, and the fourth image in Fig. 6D superimposes the second and third image to show co-localization of the channel with the endoplasmic reticulum marker. When M1766L was placed in the [H558R;Q1077del] background, the channel is rescued and makes it to the cell periphery (Fig. 6E), consistent with previous data and also showing that the rare variant L6181 in hHlb was not responsible for "rescuing" the trafficking defect.